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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)		
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Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

TUMOR SUPPRESSOR BRCA1-BARD1 UBIQUITINATES NUCLEOPHOSMIN AND IS INHIBITED BY CDK2

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ENCLOSED APPLICATION PARTS (*check all that apply*)

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☒ Applicant claims small entity status. See 37 CFR 1.27.
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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Tumor suppressor BRCA1-BARD1 ubiquitinates nucleophosmin and is inhibited by CDK2

BACKGROUND OF THE INVENTION

BRCA1, which is a tumor suppressor gene for breast and ovarian cancers, is one of the most important genes in the field of breast cancer research. Recently, the present inventors have found that BRCA1 and BARD1 form a RING heterodimeric ubiquitin ligase and that the activity of this ligase is completely eliminated by a missense mutation in *BRCA1* which causes familial breast cancer [1,4,9]. Further, the inventors have reported that polyubiquitin chains catalyzed by BRCA1-BARD1 are not the conventional Lys^{48}-linked ubiquitin chains which function as a signal for proteolysis by 26S proteasome, but Lys^{6}-linked ubiquitin chains and that these chains are de-ubiquitinated *in vitro* by 26S proteasome [2,3]. However, it has not been elucidated how the activity of the ubiquitin ligase is involved in the tumor suppressing function of BRCA1. The major reason is that the substrate for the ligase activity has not been identified.

SUMMARY OF THE INVENTION

The breast and ovarian tumor suppressor BRCA1 forms a heterodimeric RING-type ubiquitin ligase with BARD1 to catalyze untraditional Lys-6-linked polyubiquitin chains [1-4]. It is not clear how the BRCA1-BARD1 ligase regulates various cellular processes such as DNA repair, cell-cycle progression and centrosome duplication[4-6]. Here we report that BRCA1-BARD1 interacts with and catalyzes the polyubiquitination of nuclear phosphoprotein nucleophosmin/B23 (NPM), which results

in NPM stabilization. NPM co-localizes with BRCA1 at spindle poles during M phase and is ubiquitinated predominantly in cells exiting mitosis. Intriguingly, CDK2-Cyclin E and CDK2–Cyclin A, which are known to phosphorylate NPM and subsequently dissociate it from centrosomes during the G1/S transition and S phase[7,8], also phosphorylate BARD1. This results in BRCA1-BARD1 dissociation and, thus, inhibition of BRCA1-BARD1-mediated NPM ubiquitination. This novel CDK2-BRCA1-NPM pathway suggests a mechanism behind BRCA1's role in centrosome duplication.

In one aspect of the invention, a method of polyubiquitinating a nucleophosmin comprising reacting the nucleophosmin with BRCA1-BARD1 is provided. In this method, the polyubiquitination may be carried out *in vitro* or *in vivo*.

In another aspect of the invention, a method of inhibiting polyubiquitination of nucleophosmin comprising phosphorylating a BARD1 using CDK2-cyclin E and/or CDK2-cyclin A is provided. In this method, phosphorylation sites of the BARD1 may be S148, S288 and T299.

BRIEF DESCRIPTION OF DRAWING

Figure 1

Two different screens for substrates ubiquitinated by the BRCA1-BARD1 ligase identified NPM. a, Flag-ubiquitin-protein conjugates were produced from Myc-BRCA1(1-772)-BARD1 immunocomplexes as described in Materials and Methods. A portion of the reaction supernatant was analyzed by immunoblot using anti-Flag antibody (left panel) while the remainder was subjected to LC/MS/MS as summarized. **b,** Anti-Flag immunocomplexes precipitated from 293T cells expressing HA-BARD1 (1-408)

and either wild-type or I26A mutant of Flag-BRCA1 (1-222) were resolved by SDS-PAGE and stained with Sypro Ruby. The protein migrating at approximately 38-40 kDa (arrow) was digested and subjected to LC/MS/MS. **c**, NPM interacts with BRCA1-BARD1. 293T cells were transfected with indicated plasmids. Total cell lysates (upper two panels) or immunoprecipitates (IP) were subjected to immunoblotting (IB) with indicated antibodies. Anti-HA/Myc designates immunoblotting with anti-HA antibody followed by reprobe with anti-Myc antibody. [*] and [**] indicate Myc-BRCA1(1-772) and HA-BARD1, respectively.

Figure 2

NPM ubiquitination by BRCA1-BARD1. a, 293T cells transfected with indicated plasmids were boiled in 1% SDS lysis buffer, diluted to 0.1 % SDS, and immunoprecipitated with anti-Flag antibody followed by immunoblot with anti-HA antibody (upper), or anti-Flag antibody (bottom). Arrowheads indicates the migration position of non-ubiquitinated Flag-NPM. **b**, *In vivo* ubiquitinated Myc-p53 and Flag-NPM were detected as described in (**a**). **c**, Ubiquitinated endogenous NPM was immunoprecipitated from 293T cells with 1.5 μg of anti-NPM antibody and immunoblotted with anti-HA antibody as described in (**a**). **d**, Bacterially purified His-Flag-NPM was incubated in the presence of ATP with E1, UbcH5c, bovine ubiquitin (Ub), His-BRCA1 (1-304), and His-BARD1 (14-189) as indicated. The reactions were resolved by SDS-PAGE followed by immunoblot with anti-Flag antibody. Arrowheads indicate ubiquitinated Flag-NPM. [*] indicates IgG.

Figure 3

Colocalization of NPM with BRCA1-BARD1 and NPM ubiquitination in mitosis. a-c, Proliferating Swiss 3T3 cells (**a**) or COS7 cells (**b, c**) were fixed with 3% formalin or cold methanol, respectively. They were stained with indicated antibodies, followed by FITC (green)- or Rhodamine (red)- conjugated secondary antibodies. The nucleus was stained with TO-PRO-3 (blue). Merge indicates images of the two proteins (**a**) and the nucleus (**b, c**) overlaid. **d**, Hela cells were arrested at G2/M boundary by thymidine-nocodazol block and released for the indicated length of time. DNA content monitored by flow cytometry are shown at the top. The synchronized cells were boiled in 1% SDS buffer, diluted, and immunoprecipitated with 1.5 µg/ml of anti-NPM antibody, followed by immunoblot with anti-ubiquitin antibody. The nitrocellulose membrane was boiled before immunoblotting to allow antibody recognition.

Figure 4

Cdk2-Cyclin E and CDK2-Cyclin A inhibit the ubiquitination of NPM by phosphorylating BARD1 and disrupting BRCA1-BARD1 heterodimer formation. a-**c**, 293T cells were transfected with indicated plasmids. K2/E: CDK2-Cyclin E, K2/A: CDK2-Cyclin A, K1/B: CDC2-Cyclin B. *In vivo* ubiquitinated products by BRCA1-BARD1 were detected as described in Fig. 2a with the exception that CDK-Cyclins were coexpressed (**a-c**), the phosphorylation site mutant of Flag-NPM was used instead of wild-type (**b**), or anti-Myc antibody was used for immunoprecipitation to detect autoubiquitinated Myc-BRCA1 (**c**). **d**, CDK2-Cyclin E and CDK2-Cyclin A disrupt the BRCA1-BARD1 heterodimer formation. 293T cells were transfected with indicated

plasmids, immunoprecipitated with anti-Myc (upper) or anti-HA (lower) antibody and immunoblotted with anti-HA antibody. **e**, 293T cells were transfected with Myc-BRCA1(1-772), HA-BARD1 (1-320), and parental pcDNA3 vector (lane 1) or CDK2-Cyclin E (lanes 2 and 3). HA-BARD1 was immunoprecipitated with anti-HA antibody, incubated with alkaline phosphatase (AP, lane 3) or buffer alone (lanes 1 and 2) and immunoblotted with anti-HA antibody. HA-BARD1^{-P} indicates phosphorylated HA-BARD1. **f**, Bacterially purified proteins indicated at the top (4 μg each) were resolved by SDS-PAGE and stained by Coomassie Brilliant Blue (left panel). For assay, each protein (2 μg) was incubated with anti-CDK2 immunocomplexes precipitated from CDK2- and Cyclin E-overexpressing 293T cells and [γ-^{32}P] ATP, resolved by SDS-PAGE and autoradiographed. **g**, IP-Western was performed as described in (**d**) except that unphosphorylated, mutant HA-BARD1 S148A/S288A/S299A was used in lanes 4 to 6. [*] indicates IgG.

Figure 5

NPM ubiquitination by BRCA1-BARD1 is not a signal for proteasomal degradation.

a, 293T cells in 6-well plates were transfected with plasmids encoding Flag-NPM (lane 1-4, 0.5 μg) and increasing amounts of Myc-BRCA1$^{1-772}$ and HA-BARD1 (lane 2, 0.05 μg; lane 3, 0.25 μg; lane 4, 1 μg). Total plasmid DNA was adjusted to 2.5 μg per well by adding the parental pcDNA3 vector. The steady state level of each protein was analyzed by immunoblot using indicated antibodies. **b**, 293T cells transfected with Flag-NPM and indicated plasmids were incubated with cycloheximide (10 μM) and chased for the indicated lengths of time. Cell lysates were immunoblotted with anti-Flag antibody. **c**, *In*

vivo BRCA1-BARD1-ubiquitinated Flag-NPM was detected as described in Fig. 2b from cells treated with either MG132 (20 μM), LLnL (20 μM) or DMSO solvent for 10 hours before harvesting.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have identified nucleophosmin/B23/No38 (NPM) as the substrate for BRCA1-BARD1 ubiquitin ligase by two screening methods using a mass spectrometer (LC/MS/MS). NPM bound to BRCA1-BARD1 *in vivo* and was polyubiquitinated. NPM was also ubiquitinated *in vitro* by BRCA1-BARD1. This polyubiquitination did not function as a signal for 26S proteasome-dependent proteolysis. On the contrary, NPM was stabilized by BRCA1-BARD1 *in vivo*. In immunocytostaining experiments, NPM was present in the nucleolus and BRCA1 and BARD1were present within the nucleus other than the nucleolus during the interphase. However, during the mitotic phase, they co-existed around the nucleus near the spindle body and in the centrosome (spindle pole). In HeLa cells synchronized by thymidine-nocodazole block at the mitotic phase of the cell cycle, NPM was polyubiquitinated in a short term during the transition from the mitotic phase to the G1 phase.

It has been reported that NPM is present in the centrosome during the G1 phase and that centrosome replication begins when NPM has been phosphorylated by CDK2-cyclin E during the G1/S transition phase and separated from the centrosome. Since NPM returns to the centrosome at the time of mitosis and the centrosome of the daughter

cells after division has NPM, it has been indicated that NPM may be a licensing factor for centrosome replication. However, it has not been elucidated how NPM returns to the centrosome during the mitotic phase. On the other hand, it is known that deletion of BRCA1 causes excessive replication of the centrosome, which makes the genome unstable. This mechanism has not been elucidated yet.

In order to elucidate the mechanism by which NPM is polyubiquitinated during the mitotic phase, the inventors have analyzed the effects of CDK2-cyclin E and CDK2-cyclin A upon the polyubiquitination of NPM by BRCA1-BRD1. Interestingly, CDK2-cyclin E and CDK2-cyclin A completely inhibited the polyubiquitination of NPM by BRCA1-BRD1 *in vivo*. This inhibition was resulted from the phosphorylation of $S^{148}/S^{288}/T^{299}$ of BARD1 by CDK2-cyclin E and CDK2-cyclin A. BRCA1 and BARD1 were dissociated by this phosphorylation and, as a result, the ubiquitin ligase activity was completely eliminated.

These results revealed that CDK2-BRCA1-NPM pathway exists as a control mechanism for centrosome replication and suggested a possibility that the ubiquitination of NPM by BRCA1-BARD1 may play an important role in the re-localization of NPM to the spindle pole during the mitotic phase.

Targeted disruption of the *Brca1* gene in mouse resulted in centrosome hyper-amplification and genomic instability[13, 14]. During mitosis BRCA1 is localized to the centrosome via its binding to gamma-tubulin [15, 16]. It is noteworthy that NPM also

redistributes to the spindle poles during mitosis[17]. Ubiquitination of NPM by BRCA1-BARD1 could be important for this redistribution, and its defect may cause the centrosome hyper-amplification. Furthermore, many functions attributed to NPM overlap with those of BRCA1 including its upregulation after DNA damage[18], a role during the cell cycle and apoptosis[19, 20] and implications in chromatin remodeling[21, 22]. Ubiquitination of NPM by BRCA1-BARD1 may be one mechanism contributing to BRCA1's known functions. Finally, Cyclin E expression at the protein level is severely altered in sporadic breast cancers, and its high expression level is significantly correlated with a poor prognosis [23]. In light of this report, the interpretation of high Cyclin E levels would be dampening of BRCA1 ubiquitin ligase activity. Thus, ubiquitin ligase activity of BRCA1 appears to be involved in sporadic breast cancers and familial breast cancers.

EXAMPLES

Method

Antibodies, expression constructs and purified proteins

Mouse monoclonal antibodies to HA (12CA5, Boehringer, Mannheim), Myc (9E10, BabCo), Flag (M2, Sigma), polyubiquitin (Affiniti), α- and β-tubulin (DMIA+BMIB, Neomarkers), and NPM (Sigma-Aldrich or Zymed) as well as rabbit polyclonal antibodies to BRCA1 (Santa Cruz c-20) were purchased commercially. Rabbit polyclonal antibody made against the C-terminus of BARD1 was generated to the synthesized peptide CVMSFELLPLDS and affinity purified before use. Its specificity for immunofluorescence in cells was confirmed by using the competing peptide.

cDNA for full length human NPM (B23.1) was amplified by PCR from a HeLa cell cDNA library using Pfx polymerase (Stratagene). It was subcloned into the mammalian expression pcDNA3 vector in frame with the N-terminal Flag tag or into the bacterial expression pET vector in frame with the N-terminal 6xHis-Flag tag. Mammalian expression plasmids for BRCA1, BARD1 and HA-ubiquitin were previously described[1,2]. The point mutations including that for a stop codon to make truncated mutants were produced by site-directed mutagenesis (Stratagene). All plasmids used were verified by DNA sequencing. Mammalian expression plasmids for cyclins and CDKs were gifts from Dr. Yue Xiong (University of North Carolina at Chapel Hill).

Rabbit E1 (Affiniti Research Products), bovine ubiquitin (Sigma) and Flag-ubiquitin (Sigma) were purchased commercially. His-UbcH5c, and truncated N-terminal fragment of His-BRCA1 and His-BARD1 were previously described[1]. Full length His-Flag-NPM was obtained by two-step purification using nickel agarose beads followed by anti-Flag cross-linked agarose beads. The purified proteins were stained with Coomassie Brilliant Blue, and their concentrations were determined by comparison to protein standards using a densitometer (LAS 3000, Fuji film).

Cell culture, transfection and immunological techniques

Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (293T, HeLa and COS7) or 10% newborn calf serum (3T3 Swiss) and 1% antibiotic-antimycotic agent (Life Technologies, Inc). Cells were transfected using the standard calcium phosphate precipitation method as previously described[24]. To

analyze the effect of proteasome inhibitors, cells were treated with MG132 (20 μM), LLnL (20 μM) or the same volume of DMSO solvent (1 μl/ml culture) for 10 hours before harvesting. Cell cycle synchronization by thymidine-nocodazole was previously described[25]. The cells were then stained with propidium iodide and subjected to flow cytometry using FACSCalibur (Becton Dickinson).

Immunoprecipitation and immunoblotting methods, including the detection of *in vivo* ubiquitinated substrates, were previously described[1,2] except that Flag peptide was used to elute Flag-NPM from anti-Flag antibody-conjugated agarose beads (M2, Sigma) according to the manufacturer's instructions. The *in vitro* ubiquitin ligation assay was performed as previously described[1,2] except that His-Flag-NPM (0.5 μg) was added to the reaction. *In vitro* kinase assay was performed as described[26]. For dephosphorylation of immunoprecipitated HA-BARD1, HA-BARD1 immobilized beads were incubated with 2U of calf intestinal alkaline phosphatase (Takara) at 37 °C for 30 minutes.

Screening using mass spectrometry

The ubiquitin ligation reaction was performed using Flag-ubiquitin, E1, His-UbcH5c and anti-Myc immunocomplexes immobilized on protein A agarose beads precipitated from 293T cells expressing Myc-BRCA1 (1-772) and BARD1 as described[2]. The supernatant from ten reactions (30 μl each) was collected and incubated with a 30 μl volume of anti-Flag antibody-cross linked beads (Sigma). The proteins conjugated with Flag-ubiquitin were eluted off the beads in 30 μl of 25 mM anmonium bicarbonate containing 0.1 mg/ml of Flag peptide and digested with 7.4 μg/ml of trypsin for 20 hours at 30°C.

Alternatively, proteins interacting with the complex of HA-BARD1(1-408) and either wild-type or mutant (I26A) Flag-BRCA1(1-222) were immunoprecipitated and eluted as described above, resolved by SDS-PAGE, and stained with Sypro Ruby (Molecular Probe) according to the manufacturer's instructions. The bands of interest were excised from the gel and digested with trypsin using the In Gel Digest Kit (Millipore) according to the manufacturer's instructions.

The peptide fragments were subjected to LC/MS/MS analysis as described[2]. The acquired collision-induced dissociation spectra were analyzed by Mascot software.

Indirect immunocytochemistry

Proliferating cells were fixed with 3% formalin for 15 minutes and permeabilized with 0.2% Triton X-100 for 5 minutes. Cells were washed with phosphate-buffered saline (PBS), blocked with 0.5% BSA in PBS, and stained with indicated antibodies. For detection of BRCA1 and NPM colocalization at spindle poles, cells were fixed with cold methanol and permeablized with 0.1% Triton X-100 buffer as described elsewhere[15]. Primary antibodies were diluted in the blocking buffer at the following concentrations: anti-NPM (0.5 μg/ml); anti-α/β-tubulin (1 μg/ml); anti-BARD1 (3 μg/ml); and anti-BRCA1 (2 μg/ml). FITC or Rhodamine-conjugated secondary antibodies (Jackson Immunoresearch) were used at 1:50 dilution. The nucleus was counterstained with 0.5 μM TO-PRO-3 (Molecular probe), cells were then mounted with fluorescent mounting medium (BioLad) and examined with a confocal laser scan microscope (LSM 510, Carl Zeiss).

Results

BRCA1 acquires significant ubiquitin ligase activity when it forms a RING heterodimer with BARD1 [1-4]. To determine the biological significance of this activity, we searched for candidate substrates using two different approaches.

Two different screens for substrates ubiquitinated by the BRCA1-BARD1 ligase identified NPM.

First, hypothesizing that ubiquitin ligase immunocomplexes may contain substrates, we performed *in vitro* polyubiquitination of BRCA1-BARD1 immunocomplexes and analyzed the polyubiquitinated products by nanoscale capillary liquid chromatography-tandem mass spectrometric (LC/MS/MS) analysis (Fig. 1a). Of the proteins identified, nucleophosmin/B23 (NPM) exhibited the highest possibility with 17 peptides displaying a probability based Mowse score of 126 (Table 1).

Second, presuming that substrate proteins would associate with a ubiquitin ligase transiently and that this association could be stabilized by the loss of ligase activity, we compared the proteins associated with wild-type BRCA1-BARD1 ligase and catalytically inactive BRCA1(I26A)-BARD1 ligase. The I26A mutation in BRCA1 retains the BARD1 interaction site, but it disrupts the interaction with UbcH5c resulting in a catalytically dead BRCA1-BARD1 heterodimer enzyme[9]. A protein of approximately 38-40 kDa was present in the mutant ligase complex at a higher level and

was identified by LC/MS/MS as NPM (Fig. 1b and Table 1). Thus, two different screening approaches identified the same protein, NPM.

The interaction between BRCA1-BARD1 and NPM *in vivo* was confirmed by transient transfection followed by immunoprecipitation (IP)-Western analysis (Fig. 1c). 293T cells were cotransfected with Myc-BRCA1(1-772), HA-BARD1 and Flag-NPM. *In vivo* association of NPM with the BRCA1-BARD1 complex was demonstrated by reciprocal IP-Western analysis (lane 4). Omitting either BRCA1 or BARD1 from the transfection abolished the interaction with NPM (lanes 1 and 2). This suggests that the NPM interaction is dependent on BRCA1-BARD1 heterodimer formation. Thus, NPM appears to physically interact with and may represent a substrate of the BRCA1-BARD1 ligase complex.

NPM ubiquitination by BRCA1-BARD1.

To determine if NPM is a substrate of the BRCA1-BARD1 ubiquitin ligase, Flag-NPM was co-expressed in 293T cells with HA-tagged ubiquitin, Myc-BRCA1 (1-772) and BARD1 (Fig. 2a). Thirty-six hours after transfection cells were collected and boiled in 1% SDS containing buffers, and Flag-NPM was immunoprecipitated. Immunoblotting of NPM precipitates resolved by SDS-PAGE using anti-HA antibody demonstrated a ladder characteristic of polyubiquitinated NPM (lane 3). Omission of Flag-NPM, HA-ubiquitin, Myc-BRCA1 (1-772), or BARD1 all abolished the NPM ladders supporting the idea of BRCA1-BARD1-dependent NPM ubiquitination. In a parallel *in vivo* ligase assay, another RING type E3 ligase, MDM2, promoted efficient ubiquitination of its known substrate, tumor suppressor p53[10], but exhibited no detectable

activity toward NPM (Fig. 2b, lanes 3 and 6). Conversely, BRCA1-BARD1 did not cause noticeable p53 ubiquitination (lanes 2 and 5). To eliminate the possibility that NPM ubiquitination was caused by misfolding due to ectopic overexpression, we examined whether endogenous NPM is also ubiquitinated by BRCA1-BARD1 (Fig. 2c). HA-tagged ubiquitin was co-expressed in 293T cells with Myc-BRCA1 (1-772) and BARD1. Endogenous NPM was immunoprecipitated, and NPM ubiquitination was analyzed by immunoblotting using anti-HA antibody. Polyubiquitinated NPM was readily detected when wildtype ligase was added (lane 4). However, NPM ubiquitination was abolished when the I26A mutation was substituted in BRCA1 demonstrating a dependence upon the ubiquitin ligase activity of BRCA1-BARD1 (lane 5).

We further tested whether BRCA1-BARD1 ubiquitinates NPM *in vitro* using a fully recombinant system (Fig. 2d). Recombinant His-Flag-NPM purified from bacteria incubated with purified ubiquitin, E1, E2/His-UbcH5c, His-BRCA1 (1-304) and His-BARD1 (14-189) resulted in two slowly migrating products detected by anti-Flag immunoblot (lane 7). Omission of substrate NPM, ubiquitin, E2/His-UbcH5c, BRCA1 or BARD1 all abolished NPM ubiquitination. This demonstrates BRCA1-BARD1-dependent NPM ubiquitination in a purified system. Therefore, we conclude that NPM is a substrate of the BRCA1-BARD1 E3 ubiquitin ligase.

NPM ubiquitination by BRCA1-BARD1 is not a signal for proteasomal degradation.

The most common function of polyubiquitination is to target substrates for 26S proteasome-dependent degradation that involves, if not requires, ubiquitin-ubiquitin ligations via Lys-48 in ubiquitin[11, 12]. However, we and others recently found that

BRCA1-BARD1 catalyzes untraditional Lys-6-linked polyubiquitin chain formations which are deubiquitinated by purified 26S proteasome *in vitro* instead of being targeted for degradation[2, 3]. We therefore tested whether BRCA1-BARD1-mediated NPM ubiquitination targets NPM for degradation by measuring its stability *in vivo* (Fig. 5). The steady state level of Flag-NPM expressed in 293T cells was increased rather than decreased by co-expression of BRCA1-BARD1 in a dose-dependent manner (Fig. 5a). Pulse-chase analysis also supports that BRCA1-BARD1 stabilizes NPM (Fig. 5b). Furthermore, treatment with proteasome inhibitors MG132 or LLnL does not enhance the amount of ubiquitinated Flag-NPM (Fig. 5c). These findings suggest that BRCA1-BARD1-mediated NPM ubiquitination affects the function of NPM through a non-proteolytic mechanism.

Colocalization of NPM with BRCA1-BARD1 and NPM ubiquitination in mitosis.

A rabbit polyclonal antibody specific to the C-terminus of BARD1 was raised and used to examine its subcellular localization and its colocalization with NPM during the cell cycle (Fig. 3). In interphase cells, NPM predominantly localizes to nucleoli while BARD1 is absent (Fig. 3a, three cells at left of each panel). In mitotic cells, NPM colocalizes with BARD1 particularly around mitotic spindle (Fig. 3a, a cell at right of each panel). We next examined the localization of BRCA1 and NPM under conditions where BRCA1 and NPM are readily detected at the spindle poles (Fig. 3b, and data not shown). BRCA1 colocalized with NPM at the spindle poles (Fig. c). This suggests a cell cycle-dependent BRCA1-BARD1-NPM interaction and potential regulation of NPM by BRCA1-BARD1 during mitosis. To determine whether mitotic association of BRCA1-

BARD1 and NPM is correlated with cell cycle-dependent NPM ubiquitination, we synchronized cells at the G2/M boundary by thymidine-nocodazole treatment followed by a release into G1 phase (Fig. 3d). Cell cycle synchrony was monitored by flow cytometry, and *in vivo* NPM ubiquitination was assessed by IP-Western analysis. NPM ubiquitination was detected immediately after the cells were released from the mitotic block but was not seen in mitotically arrested or G1 cells (timepoints 0.5 and 1 hour).

Cdk2-Cyclin E and CDK2-Cyclin A inhibit the ubiquitination of NPM by phosphorylating BARD1 and disrupting BRCA1-BARD1 heterodimer formation.

We next attempted to determine the molecular mechanism underlying mitotic BRCA1-BARD1-NPM colocalization and NPM ubiquitination. It has been reported that NPM is phosphorylated by CDK2-Cyclin E at the G1/S transition resulting in dissociation of NPM from centrosomes to allow daughter centrioles to divide[7, 8]. NPM is prevented from associating with the centrosomes during S phase while daughter centrioles mature into centrosomes and, subsequently, into two spindle poles. We investigated the possibility that CDK2 may affect NPM ubiquitination by the BRCA1-BARD1 ligase. Surprisingly, both CDK2-Cyclin E and CDK2-Cyclin A completely abolished the ubiquitination of NPM by BRCA1-BARD1 *in vivo* (Fig 4a, lanes 3 and 4). In the same assay, CDC2-Cyclin B was not able to interfere with BRCA1-BARD1-mediated NPM ubiquitination indicating a specific function for CDK2 in inhibiting the BRCA1-BARD1 ligase (lane 5).

There are two possible mechanisms by which CDK2 could inhibit BRCA1-BARD1-mediated NPM ubiquitination: 1) CDK2 phosphorylates NPM to disguise the

BRCA1-BARD1 recognition elements, or 2) CDK2 directly phosphorylates and inhibits the BRCA1-BARD1 ligase. To distinguish between these two models, we first tested whether the BRCA1-BARD1 ligase is capable of ubiquitinating NPM (T199A), a mutant that abolishes the CDK2 phosphorylation site [8] (Fig. 4b). NPM (T199A) remained a viable substrate of the BRCA1-BARD1 ligase (lane 1), and it remained sensitive to CDK2 inhibition (lane 2). This suggests that inhibition of NPM ubiquitination by CDK2 is not due to NPM phosphorylation. We then examined the effect of CDK2-Cyclin E and CDK2-Cyclin A on the intrinsic ligase activity of the BRCA1-BARD1 heterodimeric complex by measuring BRCA1 autoubiquitination. Both CDK2-Cyclin E and CDK2-Cyclin A completely abolished the autoubiquitination of BRCA1 whereas CDC2-Cyclin B did not (Fig. 4c). Remarkably, both CDK2-cyclin E and CDK2-cyclin A disrupted the heterodimer formation of BRCA1-BARD1 (Fig. 4d). We noticed that coexpression with either CDK2-Cyclin A or CDK2-Cyclin E retarded the mobility of BARD1 (Fig. 4d, lanes 3 and 4) and that alkaline phosphatase treatment of BARD1 immunoprecipitates restored its mobility (Fig. 4e, lane 3). This suggests that BARD1 is a direct substrate of CDK2 kinases. Furthermore, purified recombinant His-BARD1 (14-189) and His-BARD1 (1-320) can be phosphorylated by CDK2-Cyclin E or CDK2-Cyclin A *in vitro* (Fig. 4f, lanes 5 and 6 and data not shown). Coexpression of CDC2-cyclin B also caused BARD1 phosphorylation *in vivo* but gave rise to a different pattern from that caused by CDK2 (Fig. 4d, lane 5). It also did not detectably affect its association with BRCA1 suggesting the possibility that BARD1 could be phosphorylated by a variety of cell cycle kinases during different phases of the cell cycle to elicit distinct functional consequences. We mapped three phosphorylation sites in BARD1 by mutational analyses and identified

a triple mutant, BARD1 S148A/S288A/T299A, that exhibited no detectable mobility shift. The mutant remained associated with BRCA1 when co-expressed with either CDK2-Cyclin E and CDK2-Cyclin A (Fig. 4g, lanes 4, 5 and 6). Together, these results suggest that CDK2-Cyclin E and CDK2-Cyclin A phosphorylate BARD1 and causes it to dissociate from BRCA1, thereby inhibiting BRCA1-BARD1 ligase activity toward its substrate, including NPM.

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WHAT IS CLAIMED IS:

1. A method of polyubiquitinating a nucleophosmin comprising reacting the nucleophosmin with BRCA1-BARD1.

2. The method of claim 1, wherein the polyubiquitination is carried out *in vitro* or *in vivo*.

3. A method of inhibiting polyubiquitination of nucleophosmin comprising phosphorylating a BARD1 using CDK2-cyclin E and/or CDK2-cyclin A.

4. The method of claim 2, wherein phosphorylation sites of the BARD1 are S148, S288 and T299.

ABSTRACT

The present invention provides a method of polyubiquitinating a nucleophosmin comprising reacting the nucleophosmin with BRCA1-BARD1 *in vitro* or *in vivo*. The present invention also proveides a method of inhibiting polyubiquitination of nucleophosmin comprising phosphorylating a BARD1 using CDK2-cyclin E and/or CDK2-cyclin A.

Table 1: The peptide fragments of NPM identified by screens in Fig. 1a and b.

a	b
25 ADKDYHFK	74 VTLATLK
33 VDNDENEHQLSLR	135 LLSISGK
46 TVSLGAGAK	240 GPSSVEDIKAK
74 VTLATLK	268 FINYVK
143 SAPGGGSKVPQKK	
151 VPQK	
198 DTPAKNAQKSNQNGK	
203 NAQKSNQNGKDSK	
213 DSKPSSTPRSK	
216 PSSTPRSKGQESFK	
224 GQESFK	
230 KQEK	
240 GPSSVEDIK	
251 MQASIEKGGSLPK	
264 VEAKFINYVK	
268 FINYVKNCFR	
278 MTDQEAIQDLWQWR	

Fig.1

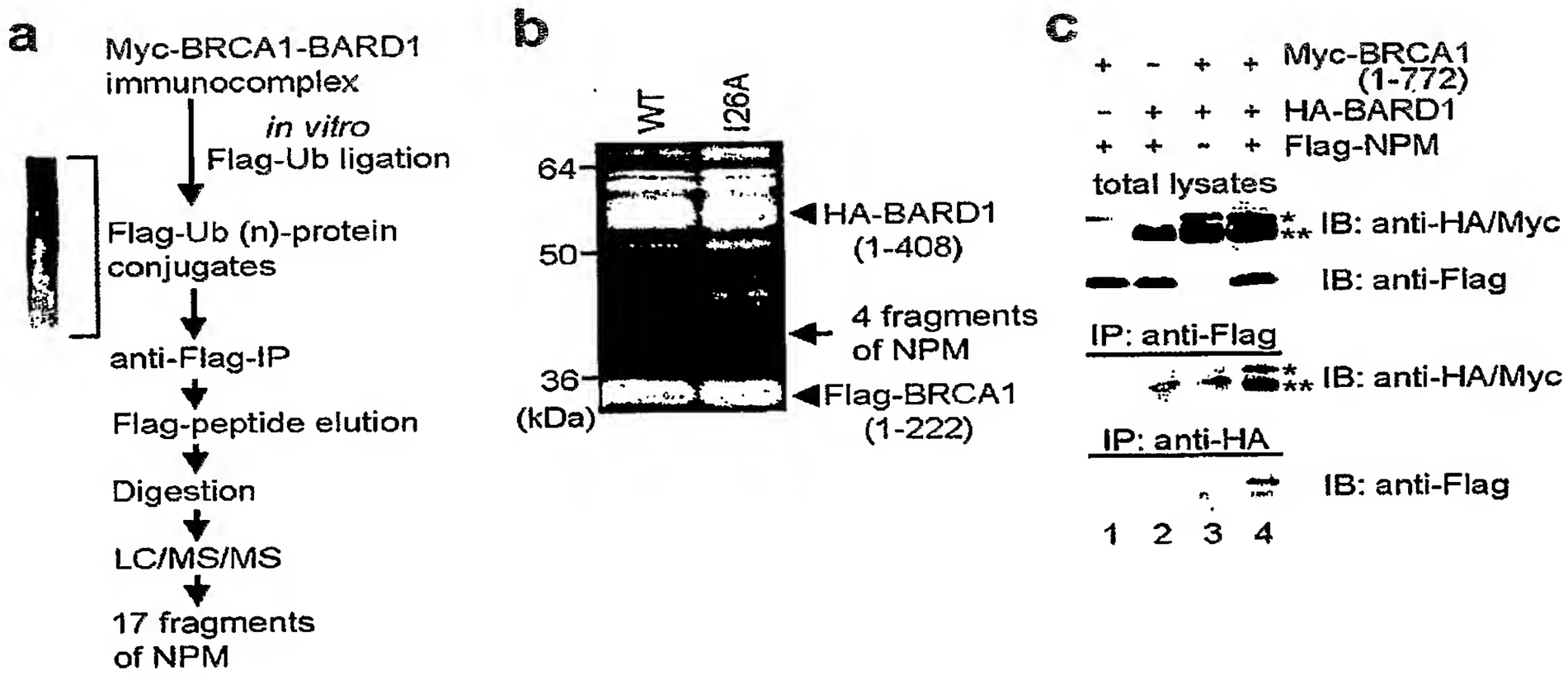

Fig.2

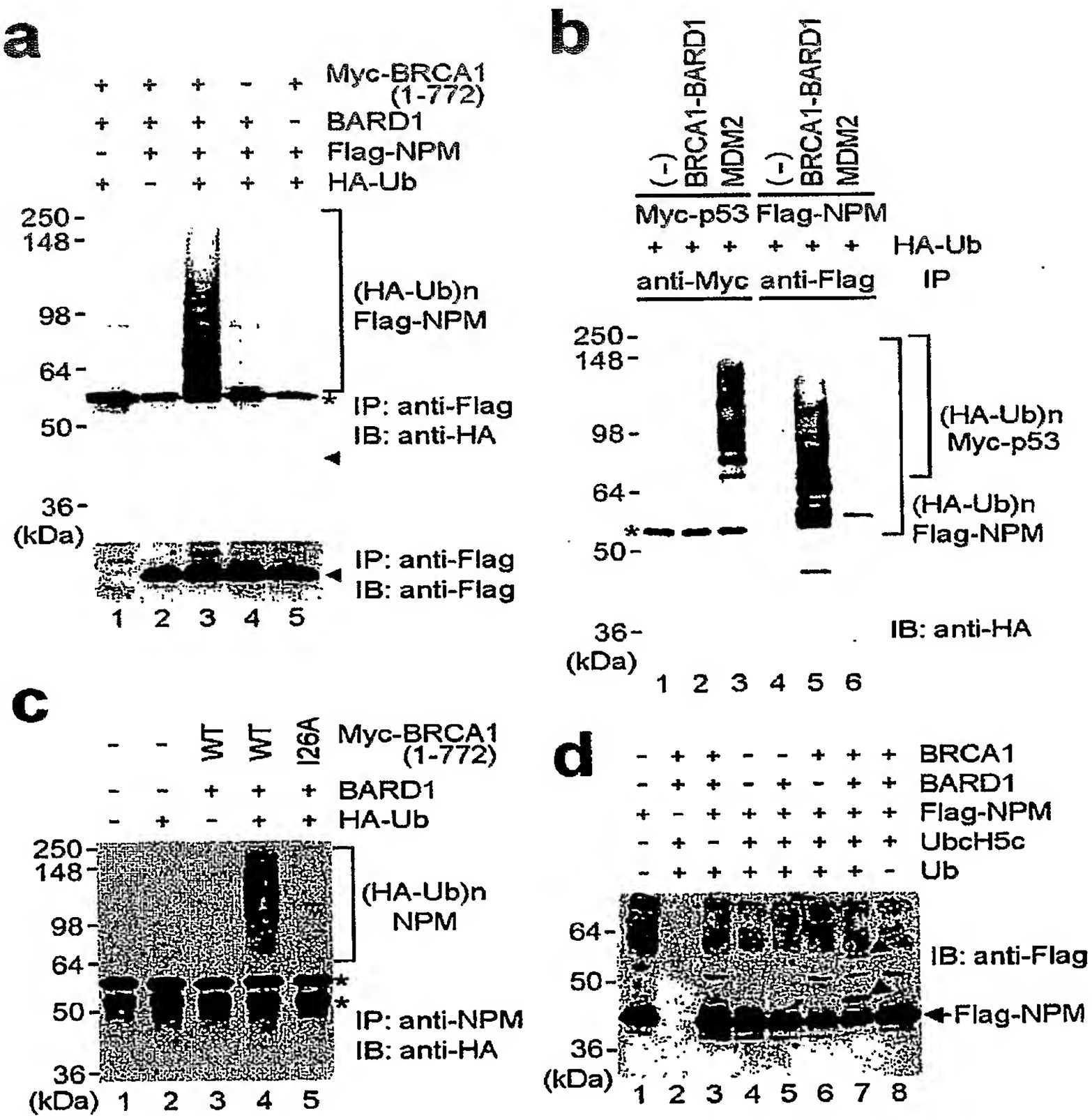

Fig.3

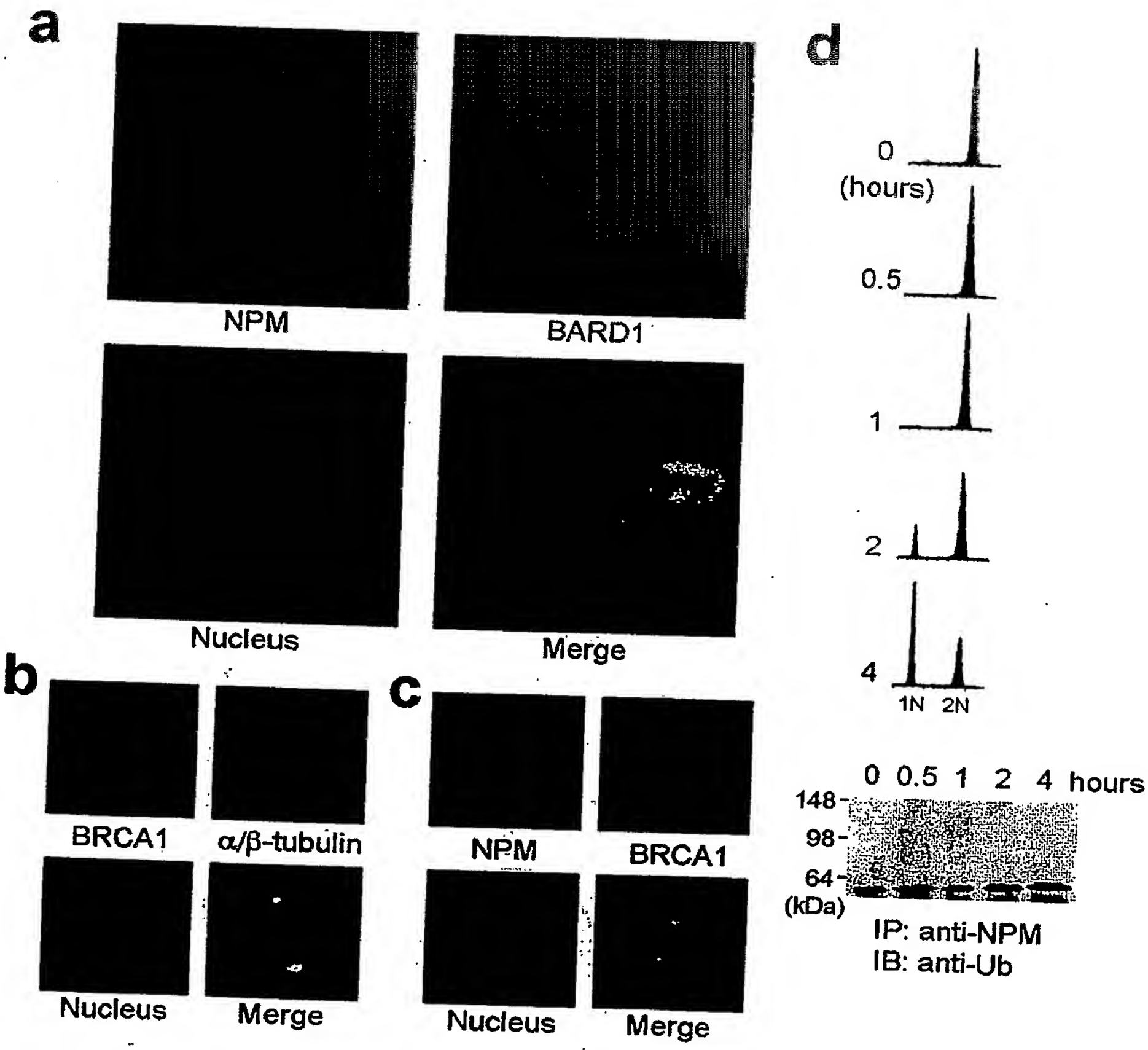

Fig.4

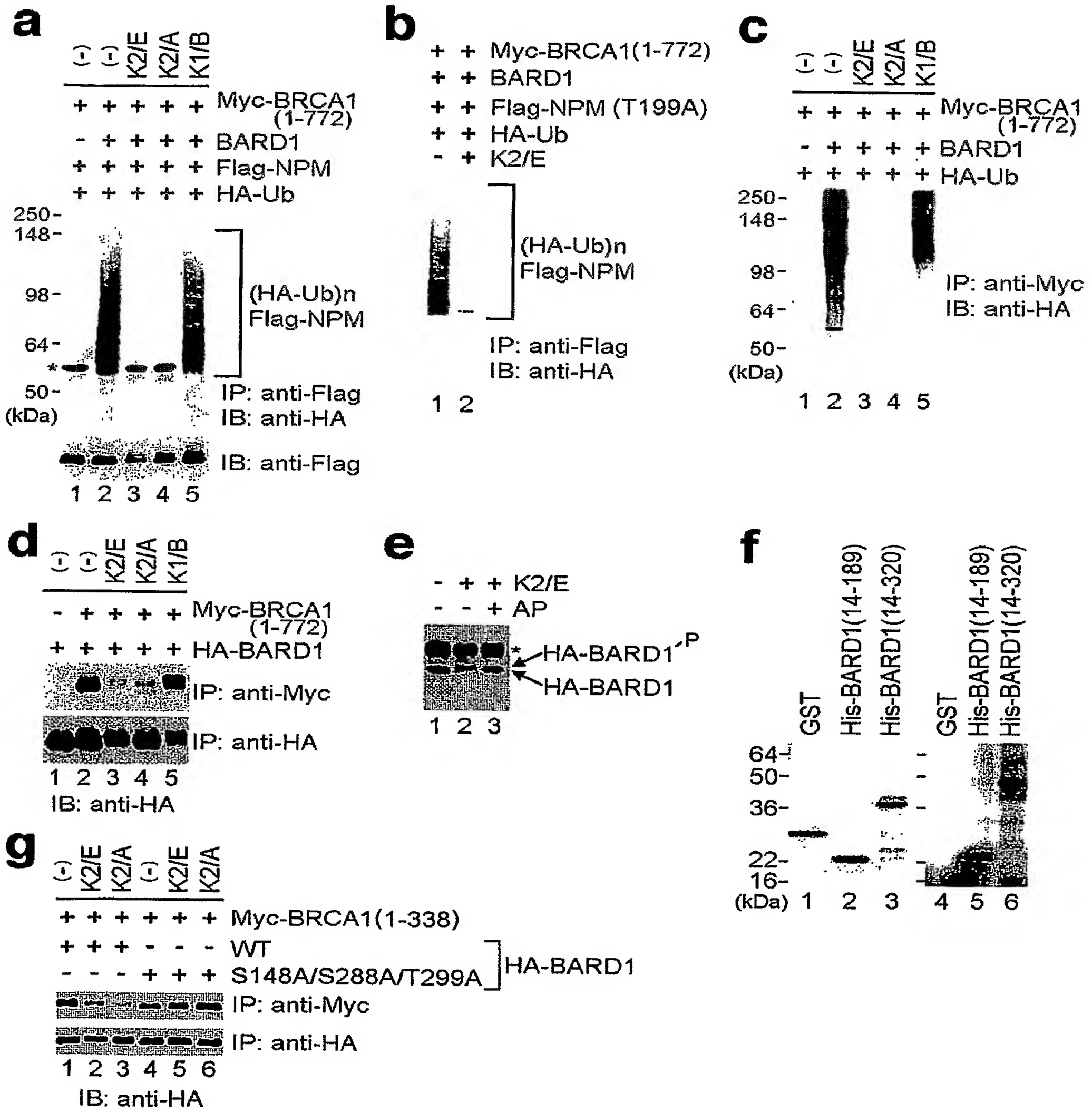

Fig.5